PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/51, C12Q 1/70 — A1

(11) International Publication Number: WO 98/00548

(43) International Publication Date: 8 January 1998 (08.01.98)

(21) International Application Number: PCT/KR97/00120

(22) International Filing Date: 25 June 1997 (25.06.97)

(30) Priority Data:
1996/24910 28 June 1996 (28.06.96) KR

(71) Applicants *(for all designated States except US)*: LG CHEMICALS CO., LTD. [KR/KR]; 20, Youido-dong, Youngdungpo-ku, Seoul 150-010 (KR). POHANG UNIVERSITY OF SCIENCE AND TECHNOLOGY FOUNDATION [KR/KR]; San 31, Hyoja-dong, Nam-ku, Pohang-si, Kyongsangbuk-do 790-784 (KR).

(72) Inventors; and

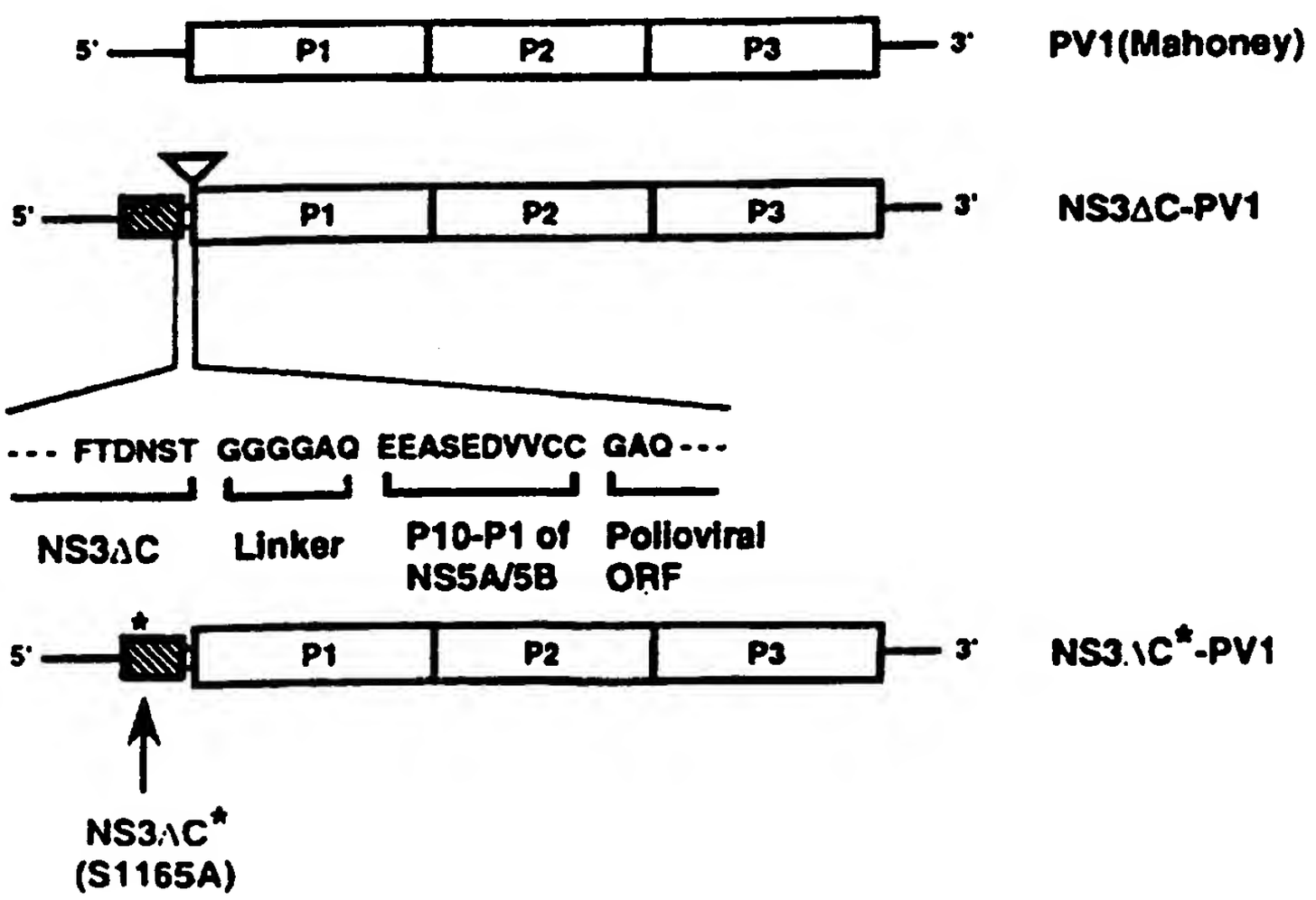
(75) Inventors/Applicants *(for US only)*: JANG, Sung, Key [KR/KR]; Kyosu Apt., 7-1002, Jigok-dong, Nam-ku, Pohang-si, Kyongsangbuk-do 790-390 (KR). HAHM, Bum, Suk [KR/KR]; Student Apt., 1-802, Jigok-dong, Nam-ku, Pohang-si, Kyongsangbuk-do 790-784 (KR).

(74) Agent: KIM, Won, Ho; You Me Patent & Law Firm, 702, Teheran Boulevard, 825-33, Yoksam-dong, Kangnam-ku, Seoul 135-080 (KR).

(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: HEPATITIS C SURROGATE VIRUS FOR TESTING THE ACTIVITY OF HEPATITIS C VIRUS PROTEASE, A RECOMBINANT GENE AND A USE THEREOF



(57) Abstract

The recombinant poliovirus which comprises poliovirus, hepatitis C virus (HCV) protease NS3 and target sites of the hepatitis C virus protease NS3, and needs the HCV protease activity, is produced, because efficient culture system for drugs against hepatitis C virus (HCV) which is the major etiologic agent of non-A, non-B hepatitis is lack.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

HEPATITIS C SURROGATE VIRUS FOR TESTING THE ACTIVITY OF HEPATITIS C VIRUS PROTEASE, A RECOMBINANT GENE AND A USE THEREOF

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a hepatitis C virus (HCV), more particularly, is related to a hepatitis C surrogate virus which comprises poliovirus genome, HCV protease and its target site, and is suitable for testing efficacy of anti-HCV drugs and screening the same, a recombinant gene and a use thereof.

Description of the Related Arts

There are known some types of viruses which cause viral hepatitis. Hepatitis A virus is an RNA virus having a diameter of 27 ㎚ and causing epidemic hepatitis through oral infection. Hepatitis B virus is a DNA virus having a diameter of 42 ㎚ and causing hepatitis through blood infection. A hepatitis virus which does not belong to any of the above types was called non-A, non-B hepatitis virus.

Hepatitis C virus (HCV) is the major etiologic agent of non-A, non-B hepatitis (Alter *et al.*, *N. Engl. J. Med.* 321, 1494-1500, 1989; Choo *et al.*, *Science*, 244, 359-362, 1989; Kuo *et al.*, *Science* 244, 362-364, 1989). This virus has been implicated in liver cirrhosis and hepatocellular carcinoma (Bruix *et al.*, *Lancet* ii, 1004-1006, 1989; Saito *et al.*, *Proc. Natl. Acad. Sci.* USA, 87, 6547-6549, 1990).

At present, α-interferon is widely used for treating HCV patients. However, only about half of the patients respond to α-interferon, and about half of the responders suffer from a recurrence of the virus (Hino *et al.*, *C. J. Med. Virol.*, 42, 299-305, 1994; Tsubota *et al.*, *Hepatology*, 19, 1088-1094, 1994). Development of other anti-HCV drugs is, therefore, necessary.

In order to develop the anti-HCV drugs, it is essential to set up HCV cultivation system to test the efficacy of the drugs. However, in vitro cultivation systems available to date are inefficient and technically difficult to manipulate (Yoo *et al.*, *J. Virol.*, 69, 32-38, 1995; Beach *et al.*, Viral Hepatitis Research Foundation of Japan, 286, 67, 1993; Shimizu *et al.*, *Proc. Natl. Acad. Sci.* USA, 89, 5477-5481, 1992). Furthermore, HCV titer is very low even in sera of hepatitis patients due to its nature of low yield. Accordingly, it is very difficult to screen or test for efficacy of antiviral drugs using HCV cultivation. Therefore, the surrogate virus will be very useful for studying HCV genes in virus replication and testing antiviral efficacy of drugs affecting HCV-encoded enzymes. Namely, the development of the surrogate virus for anti-HCV drugs is urgent.

SUMMARY OF THE INVENTION

Accordingly, the present invention is intended to overcome the above-mentioned disadvantage in the development of anti-HCV drugs and provide proper surrogate viruses for HCV suitable for the investigation of antiviral efficacy of drugs and proliferable in the HCV cultivation system.

An embodiment of the present invention is a recombinant gene coding hepatitis C surrogate virus comprising a virus gene coding picornavirus, a

protease gene coding hepatitis C virus protease NS3 which is in an open reading frame (ORF) of said picornavirus, and a target gene coding target sites of said hepatitis C virus protease NS3 which is in said open reading frame of said picornavirus.

Among the picornaviruses, the poliovirus is preferred.

The target site is preferred to be selected from the group consisting of NS5A/5B, NS4A/4B and NS4B/5A.

The hepatitis C surrogate virus protease NS3 is preferred to cleave the peptide bond between cysteine of P1 and glycine of P1' as a target site.

In another aspect, the present invention provides a screening method of anti hepatitis C virus drugs comprising the step of detecting a material which inhibits surrogate virus proliferation expressed by the recombinant gene for coding hepatitis C surrogate virus.

In another aspect, the present invention provides a measuring method of anti-hepatitis C virus drugs activity comprising the steps of proliferating the surrogate virus expressed by the recombinant gene coding hepatitis C surrogate virus in the presence of anti-hepatitis C virus agents, and measuring the proliferating ability of the hepatitis C surrogate virus.

The present invention, in another aspect, provides a hepatitis C surrogate virus comprising the recombinant gene for coding hepatitis C surrogate virus.

The poliovirus's versatility is used in developing surrogate virus suitable for investigation of HCV protease activity.

HCV belongs to the family *Flaviviridae* whose members are enveloped viruses having a positive-sense RNA genome (Francki *et al.*, *Arch. Virol* 2(Sullo.), 223, 1991; Inchauspe *et al.*, *Proc. Natl. Acad. Sci.*, 88, 10292-10296, 1991; Miller *et al.*, *Proc. Natl. Acad. Sci.*, 87, 2957-2061, 1990; Takamizawa *et al.*, *J. Virol.* 65, 1105-1113, 1991). The RNA encodes a polyprotein (-3010 amino acids) with the following gene order: 5'-C'E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3'. During and/or after synthesis, the polyprotein is processed into functional proteins by host- and virus-encoded proteases. Core protein (C) and envelope proteins (E1 and E2) are believed to compose the structural elements of the virion particle. The balance of the processed proteins is believed to function in replication processes of the virus inside the host cells. The signal peptidase on the endoplasmic reticulum is responsible for the generation of the N-terminus of E1, E2, and possibly NS2. It has been suggested that the NS2/NS3 junction is cleaved by an HCV-encoded metalloprotease, which resides between the C-terminal portion of NS2 and the N-terminal region of NS3. The cleavage of the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites depends on the activity of the serine protease NS3 (Bortenschlager *et al.*, *J. Virol.* 68, 5045-5055, 1993; Eckart *et al.*, *Biochem, Biophys. Res. Commun.* 192, 399-406, 1993; Grakoui *et al.*, *J. Virol.* 67, 2832-2843, 1993; Han *et al.*, *J. Gen. Virol.* 76, 985-993, 1995; Hijikata *et al.*, *J. Virol.* 67, 4665-4675, 1993; Tomei *et al.*, *J. Virol.* 67, 4017-4026, 1993). HCV protease NS3 is a good putative therapeutic target molecule for the development of anti-HCV drugs, since its activity is considered essential for viral proliferation. Protease activity of NS3 resides in the N-terminal part of NS3

(Bartenschlager *et al.*, *J. Virol.* 67, 3835-3844, 1994; Han *et al.*, *J. Gen Virol.* 76, 985-993, 1995; Lin *et al.*, *J. Virol.* 68, 8147-8157, 1994), and NS4A enhances the protease activity of NS3 (Bartenschlager *et al.*, *J. Virol.* 68, 3835-3844, 1994; Failla *et al.*, *J. Virol.* 68, 3753-3760, 1994; Hahm *et al.*, *J. Virol.* 69, 2534-2539, 1995; Lin *et al.*, *J. Virol.* 69, 4373-4380, 1995; Tanji *et al.*, *J. Virol.* 69, 1575-1581, 1995). Characterization of the NS3 protease using known protease inhibitors has revealed that NS3 is a chymotrypsin-like protease (Hahm *et al.*, *J. Virol.* 69, 2534-2539, 1995).

The poliovirus is the prototype of picornaviruses. The polioviral genome is composed of a positive-sense RNA molecule that encodes a single open reading frame (ORF) (Kitamura *et al.*, *Nature* (London) 291, 547-553, 1981; Racaniello *et al.*, *Proc. Natl. Acd. Sci.* USA 78, 4887-4891, 1981). Upon infection, the genomic RNA is translated into a large precursor polyprotein via the internal ribosomal entry site (IRES) in the 5'-nontraslated region of the RNA (Jang *et al.*, *J. Virol.* 62, 2636-2643, 1988; Pelletier *et al.*, *Nature* (London) 334, 320-325, 1988; Pelletiet *et al.*, *J. Virol.* 63, 441-444, 1989). The polyprotein, in turn, is processed into the mature viral structural and nonstructural proteins by the 2A, 3C, and 3CD proteases (Harris *et al.*, *Semin. Virol.* 1, 323-333, 1991; Lawson and Semler., In "Current Topics in Microbiology and Immunology" (V.R. Racaniello, Ed.), 161, 49-87, 1990). Poliovirus is one of the best studied viruses and is relatively easy to manipulate and cultivate. Production of infectious poliovirus from cDNA clones has been practiced for a decade to study functions of poliovirus proteins (Molla *et al.*, *Science* 254, 1647-1651, 1991; Van der Werf *et al.*, *Proc. Natl. Acad. Sci.*

USA 83, 2330-2334, 1986) or for use of the poliovirus as a vector in delivering foreign genes into host cells (Alexander *et al.*, *Proc. Natl. Acad. Sci.* USA 91, 1406-1410, 1994; Andino *et al.*, *Science* 265, 1448-1451, 1994; Lu *et al.*, *J. Virol.* 69, 4797-4806, 1995)

Inventors generated a hybrid poliovirus which requires the activity of HCV protease NS3 for its proliferation, on the basis of the characteristics of HCV and poliovirus. The hybrid virus is composed of poliovirus and HCV. The proteins necessary for the proliferation and infection into other cells are produced by poliovirus gene for the hybrid virus. The poliovirus gene is preceded by HCV protease and the sites cleaved by the protease to construct this hybrid virus which requires HCV protease NS3 activity for viability. This virus is composed, sequentially, HCV NS3 protease domain plus its target site, and open reading frame of poliovirus which is necessary for virus replication and virus particle formation. The activity of the enzyme NS3 protease is necessary to survive the hybrid virus.

The hybrid virus is proliferable in the cultivation system to settle the conventional problem which HCV cannot be proliferated in vitro cultivation systems. Therefore, the hybrid virus enables to develop anti-HCV drugs based on protease inhibitor easily and precisely.

DETAILED DESCRIPTION OF THE DRAWINGS

The present invention will be described with reference to the accompanying drawings, in which:

FIG. 1 is schematic diagram of the genomic organization of poliovirus type 1 (Mahoney), NS3 △ C-PV1, and NS3 △ C'-PV1.

FIG. 2 is a photograph of plaque phenotypes of PV1 (M) and vNS3 △ C-PV1.

FIG. 3 is a photograph of RT-PCT analysis of vNS3 △ C-PV1 RNA.

Lane 1, 2 and 3 depict DNAs produced by PCR or RT-PCR using as template of pNS3 △ C-PV1, (+) sense RNA and (-) sense RNA, respectively.

FIGs. 4A, 4B and 4C are photographs of proteolytic processing patterns of PV1, NS3 △ C-PV1, and NS3 △ C'-PV1 polyproteins.

FIG. 5 is one-step graph curves of PV1 (M) and vNS3 △ C-PV1. HeLa cells were infected with either PV1 or vNS3 △ C-PV1 at a multiplicity of infection of 10.

FIG. 6A to FIG. 6E are shown the genetic sequence of pNS3 △ C-PV1 according to an embodiment of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the following detailed description, only the preferred embodiment of the invention has been shown and described, simply by way of illustration of the best mode contemplated by the inventor(s) of carrying out the invention, As will be realized, the invention is capable of modification in various obvious respects, all without departing from the invention, Accordingly, the drawing and description are to be regarded as illustrative in nature, and not as restrictive.

EXAMPLE

Material and Methods

Construction of plasmids

DNA manipulations were carried out by standard methods (Sambrook *et al.*, Molecular cloning: a laboratory manual (2nd ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989). The construction of the plasmids pTHE △ 1219-1634 and pPNENPO are described elsewhere (Han *et al.*, *J. Gen. Virol.*, 76, 985-993, 1995; Alexander *et al.*, *Proc. Natl. Acad. Sci.*, USA, 91, 1406-1410, 1994). pUC19 was cleaved with XbaI, treated with Klenow, and digested with PstI. The resulting 2.6 kb fragment was then ligated to the 1.3 kb fragment of pTHEΔ1219-1634 treated with NcoI, Klenow, and PstI in succession. The result was the construct pUC19-NS3 △ C-4 △ C. pPNENPO and pUC19-NS3 △ C-4 △ C were digested with EcoRI or KpnI, respectively, and treated with Klenow enzyme. The linearized DNAs were ligated to result in the construct pPNENPO-NS3 △ C-4 △ C.

To construct pNS3 △ C-4A-PV1, a polymerase chain reaction (PCR) was performed using the following four oligonucleotides:

Primer 1: 5'-TGATATCGAATTCCGG-3';

Primer 2: 5'-ATATGAGCTCCGCACTCTTCCATCTC-3';

Primer 3: 5'-ATATGAGCTCAGGTTTCATCACAGAAAG-3';

Primer 4: 5'-CTGTGCTAGCGCTTTTTG-3'.

The primers 1 plus 2 and pPNENPO-NS3 △ C-4 △ C were used to

amplify the HCV protease NS3 △ C-4A region (PCR product 1). The PCR product 1 contains a new SacI site right after the NS4A-coding sequence. The primers 3 plus 4 and pPNENPO were used to amplify polioviral cDNA (746-2479) with a new SacI site at the beginning of the poliovirus ORF (PCR product 2). EcoRI and NheI digested pPNENPO, EcoRI and SacI digested PCR product 1, and SacI and NheI digested PCR product 2 were ligated together to generate plasmid pNS3 △ C-4A-PV1.

To construct pNS3 △ C-PV1, an oligonucleotide (5'-AGCTCCGCAGCAGACGACGTCCTCACTGGCTTCCTCTTGCGCGCCTCCTCCT CCGGTTGAGTTATCTGTGAAGAC-3') encoding the C-terminal end of NS5A (P1-P10) and primer 1 were used to amplify the HCV region encoding amino acids 1016-1215. The PCR product was phosphorylated with polynucleotide kinase and then digested with EcoRI. pNS3 △ C-4A-PV1 was cleaved with SacI, treated with T4 DNA polymerase, and then digested with EcoRI. The larger fragment of pNS3 △ C-4A-PV1 was ligated to the PCR product to generate pNS3 △ C-PV1. The junction sequences were confirmed by sequencing.

<u>Site-directed mutagenesis of the serine in the catalytic triad of the HCV protease (Ser1165Ala).</u>

Site-directed mutagenesis was carried out by the Kunkel method as previously described (Kunkel *et al.*, *Methods Enzymol.*, 154, 367, 1987). pSK1016-1650 was generated by ligation of the DNA fragments isolated from PvuII plus SalI-digested pTHE1016-1846 and SmaI plus SalI-digested pBluescript

SK(+). E. coli strain RZ 1032 (ung⁻dut⁻) was transformed with pSK1016-1650, and ssDNA was prepared from the transformant. An oligonucleotide, 5'-CTTGAAGGGCTCCGCG GGTGG-3', designed to change the serine residue in the catalytic triad of HCV protease to alanine [Ser 1165(TCG) to Ala(GCG)] was used for site directed mutagenesis. The oligonucleotide was phosphorylated with T4 polynucleotide kinase (Boehringer Mannheim) and annealed to the single stranded pSK1016-1650 DNA, extended with sequenase (USB), and ligated with T4 DNA ligase (Poscochem.) to result in covalently closed, circular, dsDNA. The ligated dsDNA was introduced into E. coli XL1-blue. The base change was confirmed by the presence of a newly generated SacII site and by sequencing. pSK1016-1650 (Ser1165Ala) was used to construct pNS3 △ C⁻-PV1 by replacing the XmaI fragment of pNS3 △ C-PV1 with the same fragment of pSK1016-1650 (Ser1165Ala).

In vitro transcription and translation

Plasmid DNAs were purified following the polyethylene glycol precipitation method. They were then linearized with appropriate restriction enzymes downstream of the translation termination codon. The linearized DNAs were extracted with phenol/chloroform and ethanol-precipitated. RNAs were transcribed from the purified DNAs with T7 RNA polymerase (Boehringer Mannheim) as described by the manufacturer. These RNA transcripts were translated in a rabbit reticulocyte lysate system (RRL) supplemented with 25% HeLa cell lysate in the presence of [^{35}S] methionine. The *in vitro* translation reactions were carried out at 30 ℃ for 8 hours. The [^{35}S]-labeled proteins were

analyzed on 15% SDS-PAGE using the buffer system described by Nicklin et al. (1987). The intensity of the autoradiographic images was enhanced by fluorography using salicylic acid. Gels were dried and exposed to Kodak XAR-5 film or Agfa Curix RP1 film.

RNA transfection

An electroporation protocol was used to transfect HeLa cell monolayers with the viral RNAs prepared *in vitro*. Cells grown to 80% confluency were harvested. After two washes, the cells were resuspended in the medium at a concentration of 5×10^6 cells/㎖. The cell suspension (1 ㎖) was mixed with 3 ㎍ of RNA and placed in a 0.4 ㎝ Gene Pulser cuvette (BIO-RAD). The cuvette was electrically pulsed at 360 volt and 960 ㎌ using the Gene Pulser transfection apparatus (BIO-RAD). Then the cells were diluted with 9 ㎖ of medium containing 10 % bovine calf serum, 5 % equine serum, and 5 % fetal calf serum.

Plaque assay and one-step growth curve

Titers of virus stocks were measured by plaque assay on HeLa cell monolayers as follows. Cells were inoculated with virus, left for 30 minutes at room temperature, and then overlaid with Eagle medium containing 0.9 % Noble agar and 5 % fetal calf serum. Viral plaques were visualized with 0.5 % crystal violet solution 60 hours postinfection.

To measure one-step growth kinetics, virus was loaded onto 3.5 ㎝ HeLa cell plates at a multiplicity of infection of 10 per cell and incubated at room temperature for 30 minutes to allow virus binding. Unbound particles were

removed by two rounds of PBS washing. The infected cells were cultured at 37 ℃ in 2 ㎖ of Eagle medium containing 10 % bovine calf serum and harvested at the time points indicated in FIG. 5. The infectivity of the cell lysate at each time point was titrated by plaque assay.

Reverse transcription PCR (RT-PCR)

Total RNA from virus-infected cells was isolated 4 hours postinfection as described by Lu et al. (1995). cDNAs were synthesized using M-MuLV reverse transcriptase and oligonucleotide primers corresponding to poliovirus nucleotides 556-570 and 789-806 (primer 5: 5'-GTGTTTCCTTTTATT-3'; primer 6: 5'-GTGTTTCCTTTTATT-3'). Primer 5 and primer 6 were used in synthesizing cDNAs from negative- and positive-sense RNA, respectively. Both of the primers were used in the PCR reaction that followed. The PCR products were analyzed on a 1.3 % agarose gel.

Labeling of newly synthesized proteins in virus-infected cells

HeLa cells were infected with poliovirus 1 (Mahoney: PV1) and vNS3 △ C-PV1 at a multiplicity of infection of 30 PFU per cell. The cells were incubated in a medium lacking methionine for 10 minutes 3 hours after infection. Subsequently, 100 μ Ci of [^{35}S] methionine was added to the medium and the incubation continued for 40 minutes. The cells were then collected and lysed. The lysate was analyzed on 15 % SDS-PAGE.

Immunoblot analysis

Virus-infected cells were lysed 4 hours postinfection. Total protein (13

μg) was resolved on a 15 % SDS-PAGE gel and transferred onto a nitrocellulose membrane (Amersham). The blot was incubated overnight at 4 ℃ in blocking solution [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5 % Tween 20, and 5 % powdered skim milk] to block nonspecific binding. The primary antibody (polyclonal antibody against HCV protease NS3) was added to the blocking solution for 3 hours. The antibody was a generous gift from Dr. R. Bartenschlager, Institute of Virology, Mainz, Germany. A horseradish peroxidase-linked anti-rabbit IgG in a 1:10,000 dilution was used as the secondary antibody. Membrane-bound antibodies were detected by enhanced chemiluminescence reagents (ECL; Amersham).

Generation of a novel poliovirus containing HCV protease NS3 and its target site

In order to develop an evaluation system for anti-HCV drugs targeting protease NS3, a recombinant poliovirus that requires the activity of HCV protease for its proliferation, was constructed. A schematic diagram of the genomic structure of this virus is depicted in FIG. 1. The hatched and open boxes represent HCV protease NS3 △ C and poliovirus polyprotein, respectively. The amino acid sequence at the junction of HCV protease NS3 and poliovirus P1 is indicated by single letter amino acid codes. The target site of HCV protease is marked by an arrowhead. The mutated amino acid (S1165A) in NS3 △ C*-PV1 is indicated by an asterisk.

As shown in FIG. 1, in the case of polyprotein NS3 △ C-PV1, the protease

domain of HCV NS3 is fused to the last 10 amino acids of NS5A (EEASEDVVCC), which are recognized by protease NS3. These amino acids residues are preceded by four glycines, alanine, and glutamine serving as a hinge region between the protease domain and the target site. It was expected that the HCV protease NS3 would cleave the peptide bond between cysteine at the C-terminus of the additional polypeptide and glycine at the N-terminus of poliovirus P1, since HCV protease NS3 is known to cleave between the cysteine or threonine at the P1-residue and the small uncharged residues at the P1'-residue (Kolykhalov *et al.*, *J. Virol.* 68, 7525-7533, 1994; Komoda *et al.*, *J. Virol.*, 68, 7351-7357, 1994).

Upon transfection, the RNA transcript encoding NS3 △ C-PV1 produced a virus designated vNS3 △ C-PV1. On the other hand, the RNA transcript similar to NS3 △ C-PV1 but containing a serine to alanine mutation at the catalytic triad of HCV protease NS3 (NS3 △ C*-PV1) did not yield virus. This strongly suggests that HCV protease NS3 activity is essential for the viability of the hybrid virus vNS3 △ C-PV1. The virus vNS3 △ C-PV1 has a small plaque phenotype at 37 ℃ as shown in FIG. 2. (compare plaque sizes in panel PV1 with vNS3 △ C-PV1) or at 30 ℃ (data not shown).

The virus vNS3 △ C-PV1 is stable through at least five passages. The integrity of vNS3 △ C-PV1 was confirmed by analyzing the viral RNAs in cells inoculated with a virus stock of the fourth passage. Lanes 1, 2 and 3 depict DNAs produced by PCR or RT-PCR using as templates plasmid pNS3 △ C-PV1, (+) sense RNA, respectively. The inserted HCV sequence in the poliovirus

genome was amplified by RT-PCT with primers annealing to the poliovirus 5' nontranslated region and the P1-coding region. The viral RNAs of the fifth passage retained the inserted HCV protease and NS5A sequences as shown in FIG. 3. Both positive and negative sense viral RNAs of the expected sizes were detected (FIG. 3, lanes 2 and 3, respectively). RT-PCR analysis of the genomic RNAs isolated from vNS3 △ C-PV1 virion particles revealed the same RT-PCR products as the RNAs isolated from HeLa cells (data not shown). The junction sequence between HCV and poliovirus was confirmed by DNA-sequencing of the PCR products and the sequences of hepatitis C surrogate virus genome is presented in FIG. 6. This indicates that vNS3 △ C-PV1 is stable at least for five passages.

Proper proteolytic processing of vNS3 △ C-PV1 polyprotein requires HCV NS3 protease activity.

Proteolytic processing patterns of polyproteins encoded by poliovirus, vNS3 △ C-PV1, and NS3 △ C'-PV1 RNA were analyzed by *in vitro* transcription and translation of pT7-PC1, pNS3 △ C-PV1, and pNS3 △ C'-PV1. Most of the polioviral proteins were detected as *in vitro* translation products of the PV1 RNA transcript (FIG. 4A, lane 1). Two additional protein bands of 123 kDa and 26 kDa were apparent among the translation products of NS3 △ C-PV1 as compared to the PV1-translation product (FIG. 4A, lane 2). The 123 kDa and 26 kDa proteins are most likely NS3 △ C-P1 fusion protein and the processed NS3 △ C, respectively, since the cleavage of poliovirus at the P1/P2 junction occurs very

fast, even during the process of translation. Translation of vNS3 △ C*-PV1 also yielded the 123 kDa protein. But the 26 kDa and the P1 protein were not detected among the translation products from this construct (FIG. 4A, lane 3). This indicates that proteolytic cleavage of the HCV/poliovirus junction does not occur in NS3 △ C*-PV1, and that the cleavage of this junction is mediated by the protease activity of NS3. It also shows that the failure of virus production with the NS3 △ C*-PV1 construct is most likely due to the lack of the P1 polypeptide which is a protein processed into viral coat protein (VP0, VP1, and VP3). In other words, HCV NS3 activity is essential for proliferation of the vNS3 △ C-PV1. Interestingly, complete processing of the viral capsid proteins (VP0, VP1, and VP3) from the NS3 △ C*-PV1 polyprotein was not detected. On the other hand, the processed capsid proteins were apparent along with the precursor NS3 △ C-P1 (compare lane 2 with lane 3 in FIG. 4A). The blockage of proteolytic processing of the NS3 △ C*-PV1 polyprotein may be due to the lack of myristoylation of the glycine residue at the N-terminus of P1 that has been shown to be important in the processing of P1 by poliovirus protease 3C-3D (Kräusslich *et al.*, *J. Virol.*, 64, 2433-2436, 1990).

The proteolytic processing was also investigated for PV1 and vNS3 △ C-PV1 by labeling newly synthesized proteins with [^{35}S] methionine in virus-infected cells. The labeling in vNS3 △ C-PV1-infected cells showed four additional bands with apparent molecular weights of 123 kDa, 89 kDa, 63 kDa and 26 kDa as indicated by arrow heads (FIG. 4B, compare lane 2 with lane 3). These proteins

are most likely NS3 △ C-P1, NS3 △ C-VP0-VP3, NS3 △ C-VP0, and NS3 △ C, respectively (FIG. 4B). This assumption is supported by the Western blotting analysis using an antibody against HCV NS3 and HeLa cell extracts after infection with either wild type poliovirus or vNS3 △ C-PV1 (FIG. 4C). Among the proteins of vNS3 △ C-PV1-infected cells, the anti-NS3 antibody recognized the 63 kDa and the 26 kDa polypeptides corresponding to vNS3 △ C-VP0 and NS3 △ C, respectively (FIG. 4C, lane 3). A longer exposure of the X-ray film also revealed the 123 kDa band and the 89 kDa band corresponding to NS3 △ C-P1 and NS3 △ C-VP0-VP3, respectively (data not shown). On the other hand, no bands were detected in the Western analysis of cell extracts from wild type poliovirus- or mock-infected cells (FIG. 4C, lanes 1 and 2). The results indicate that proteolytic processing of the artificial polyprotein occurs as expected, even though the HCV/poliovirus junction cleavage happens slower than the P1/P2 junction cleavage. The relative intensities of the NS3-related bands varied depending on the methodologies used to detect the polypeptides. For instance, a strong band of NS3 △ C-P1 was detected by *in vitro* and *in vivo* labeling, but only a weak band of NS3 △ C-P1 was detected by Western blot analysis. The [^{35}S] methionine labeling method reveals only newly synthesized polypeptides and larger proteins, containing more methionine residues, give stronger signals. Western blot analysis, on the other hand, reveals all of the proteins interacting with the primary antibody, and the intensity of the signal reflects the molarities of the proteins rather than their size. Therefore, [^{35}S]methionine labeling methods are expected

to give relatively stronger signals for precursor forms than Western blot analysis methods.

One-step growth kinetics of chimeric virus vNS3 △ C-PV1

FIG. 5 was one step growth curve for vNS3 △ C-PV1. While maximum production of wild type poliovirus was reached 4 hours postinfection (FIG. 5, open squares), that of vNS3 △ C-PV1 was delayed by about 2 hours (FIG 5, closed circles). In FIG. 5, the titer of vNS3 △ C-PV1 at the peak was about 1`.5 orders of magnitude lower than that of wild type poliovirus, as viral titers at 9 hours postinfection was compared. The small plaque phenotype of vNS3 △ C-PV1 may thus be a result of delayed virion particle formation and smaller burst size.

The HCV protease NS3 activity is necessary for noble poliovirus according to the present invention to survive. This virus is useful for testing the efficacy of anti-virus drugs which targets protease NS3.

WHAT IS CLAIMED IS:

1. A recombinant gene coding hepatitis C surrogate virus comprising:

a virus gene coding picornavirus;

a protease gene coding hepatitis C virus protease NS3 which is in an open reading frame (ORF) of said picornavirus; and

a target gene coding target sites of said hepatitis C virus protease NS3 which is in said open reading frame of said picornavirus.

2. The recombinant gene coding hepatitis C surrogate virus of claim 1, wherein said picornavirus is poliovirus.

3. The recombinant gene coding hepatitis C surrogate virus of claim 1, wherein said target site is selected from the group consisting of NS5A/5B, NS4A/4B and NS4B/5A.

4. The recombinant gene coding hepatitis C surrogate virus of claim 1, wherein said hepatitis C surrogate virus protease NS3 cleaves the peptide bond between cysteine of P1 and glycine of P1' as a target site.

5. A screening method of anti hepatitis C virus drugs comprising the step of:

detecting a material which inhibits surrogate virus proliferation expressed by said recombinant gene for coding hepatitis C surrogate virus of claim 1.

6. A measuring method of anti-hepatitis C virus drugs activity comprising the steps of:

proliferating the surrogate virus expressed by said recombinant gene coding hepatitis C surrogate virus of claim 1 in the presence of anti-hepatitis C virus drugs; and

measuring the proliferating ability of said hepatitis C surrogate virus.

7. A hepatitis C surrogate virus comprising:

a recombinant gene for coding hepatitis C surrogate virus of claim 1.

FIG. 1

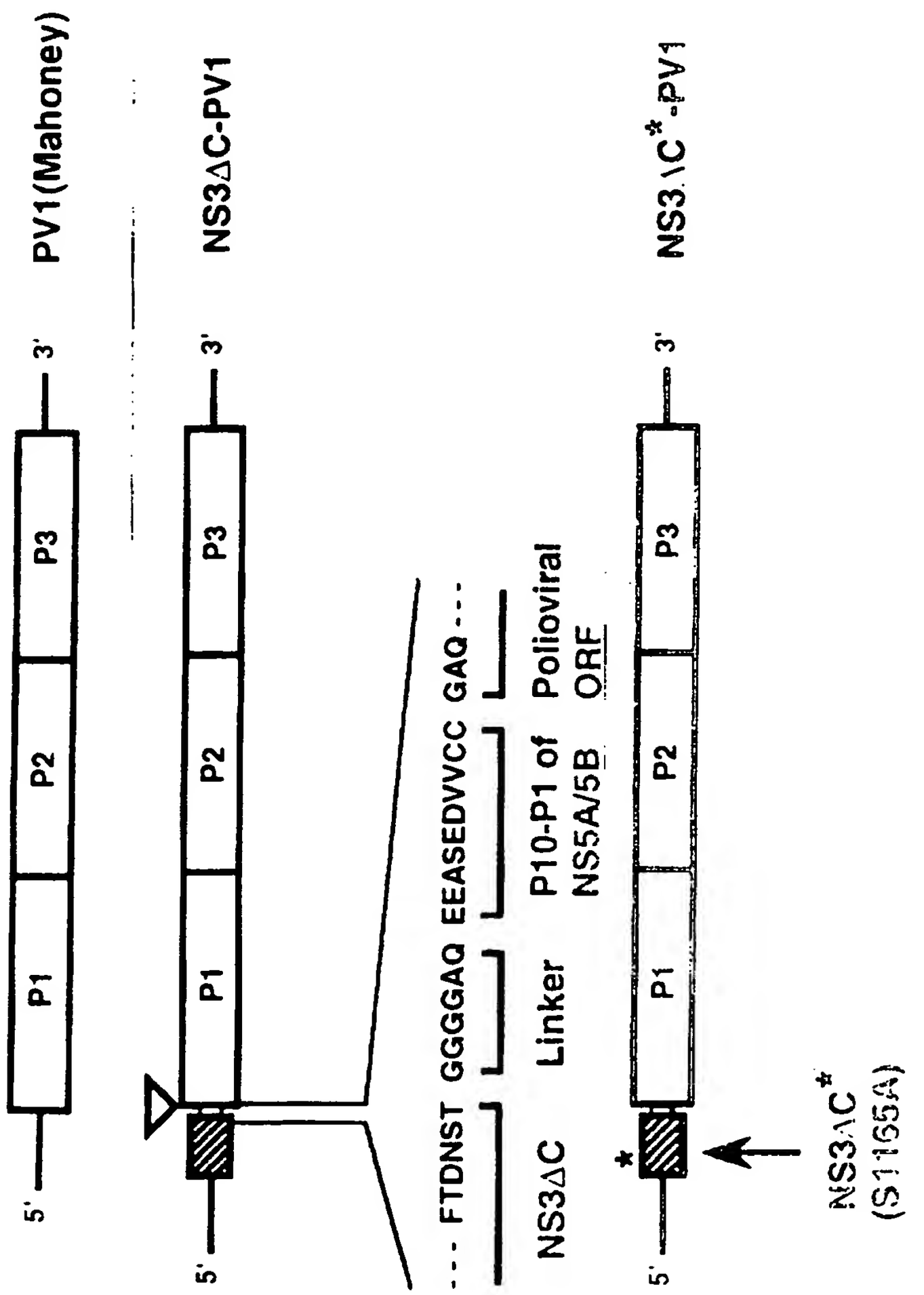
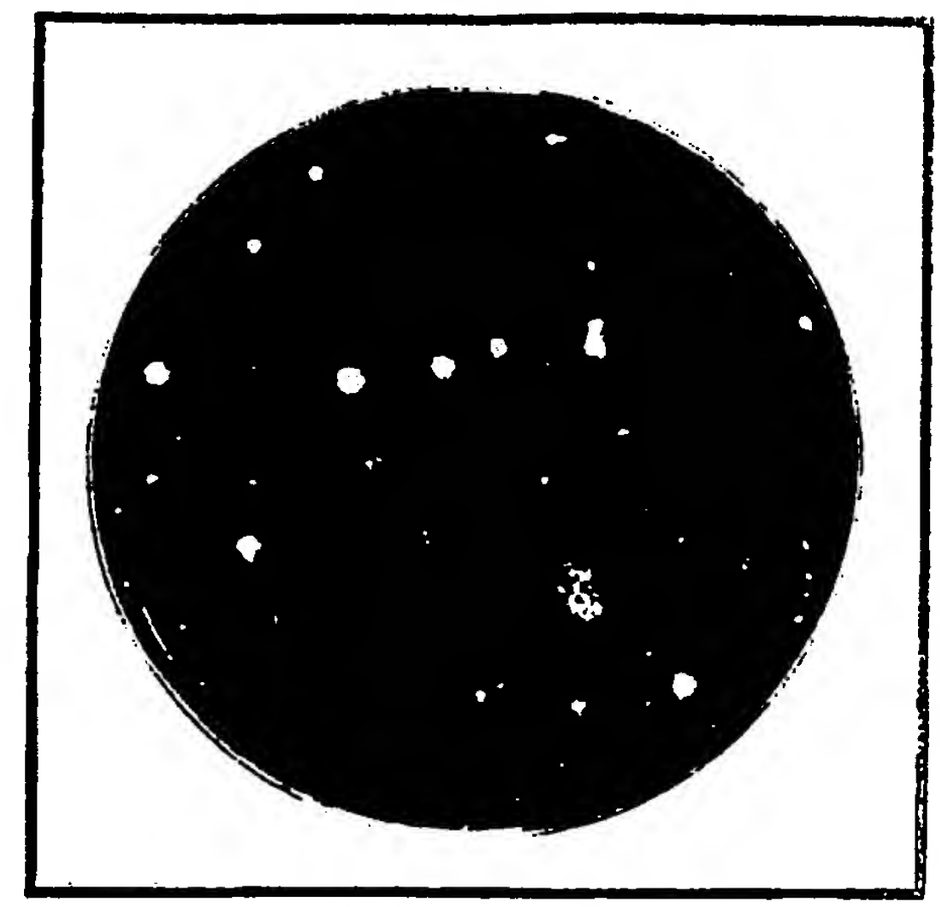
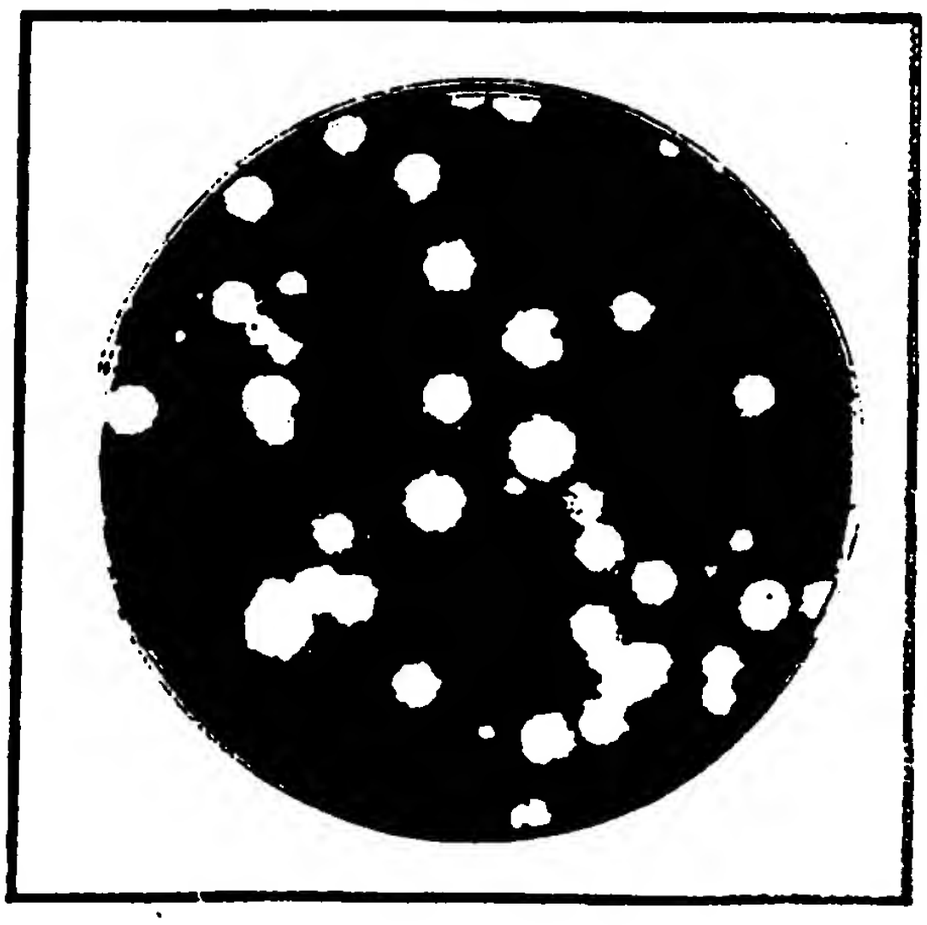

PV1(Mahoney)
P1
P2
P3
3'
5'
PV1ΔC-PV1
NS3ΔC
NS3ΔC*-PV1
--- FTDNST GGGGAQ EEASEDVVCC GAQ ---
NS3ΔC Linker P10-P1 of Polioviral NS5A/5B ORF
NS3ΔC*
(S1165A)

FIG. 2



vNS3ΔC-PV1



PV1

FIG. 3

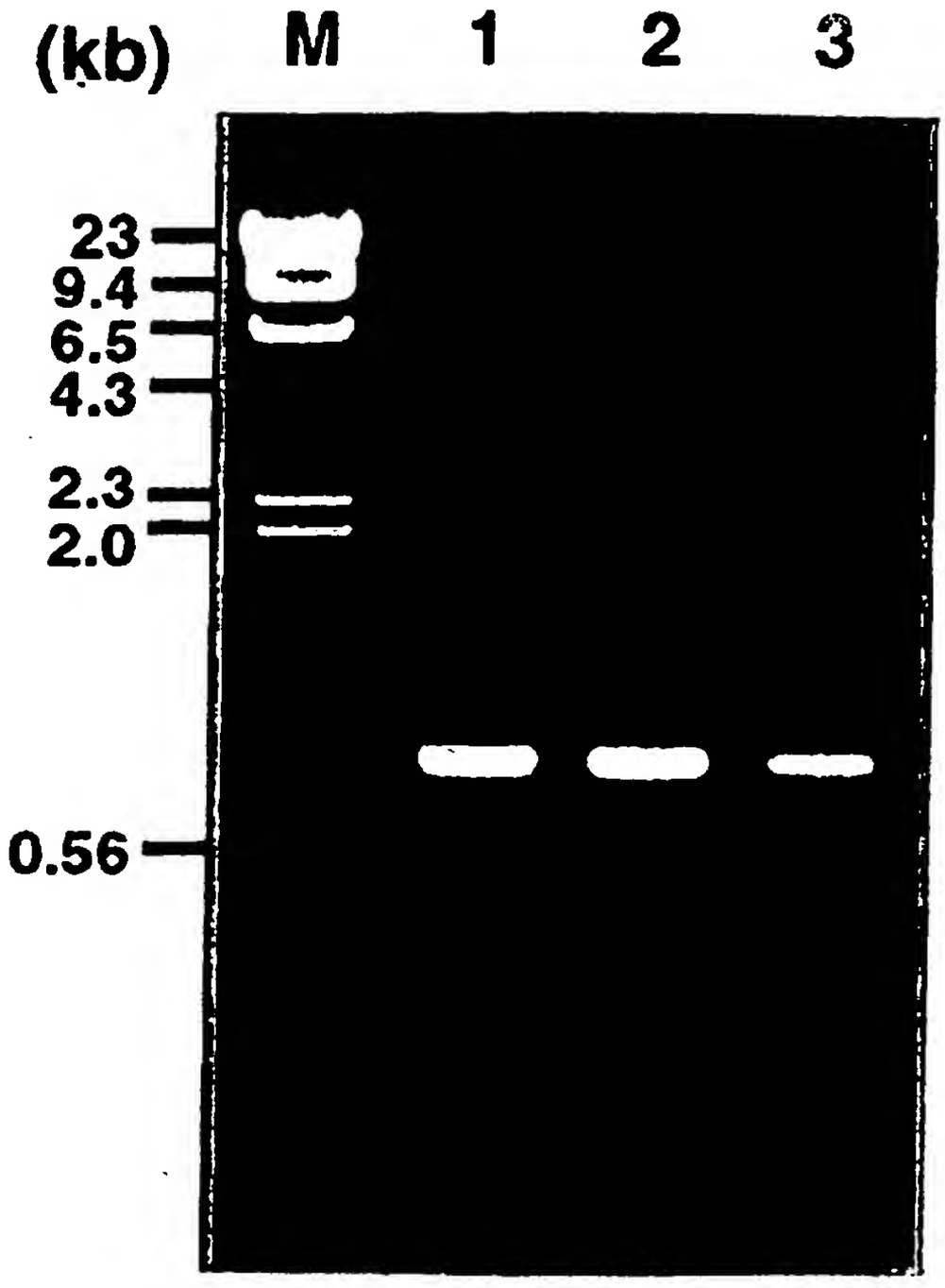


FIG. 4A

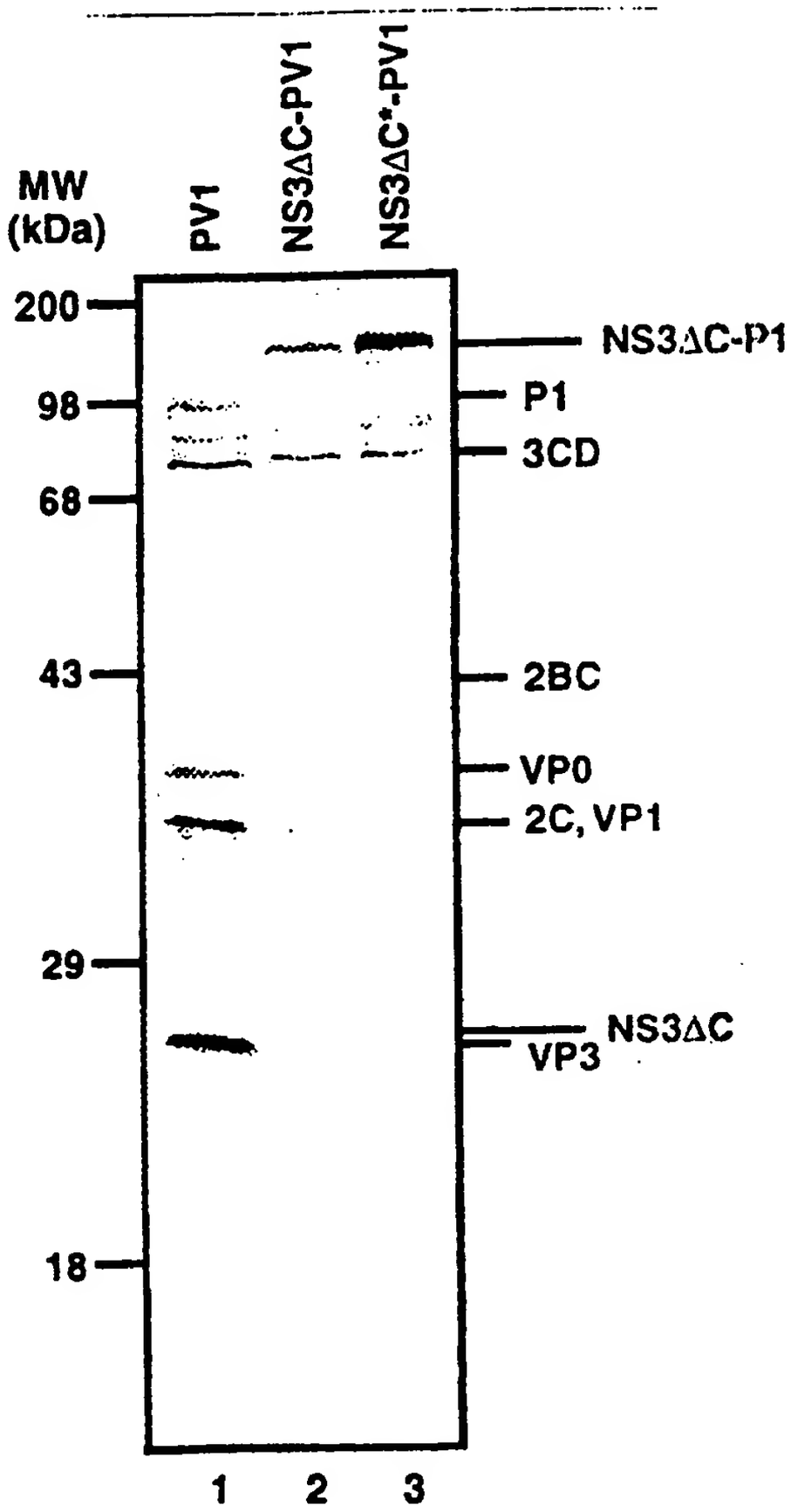

MW (kDa)
PV1
NS3ΔC-PV1
NS3ΔC*-PV1
200
98
68
43
29
18
NS3ΔC-P1
P1
3CD
2BC
VP0
2C, VP1
NS3ΔC
VP3
1
2
3

FIG. 4B

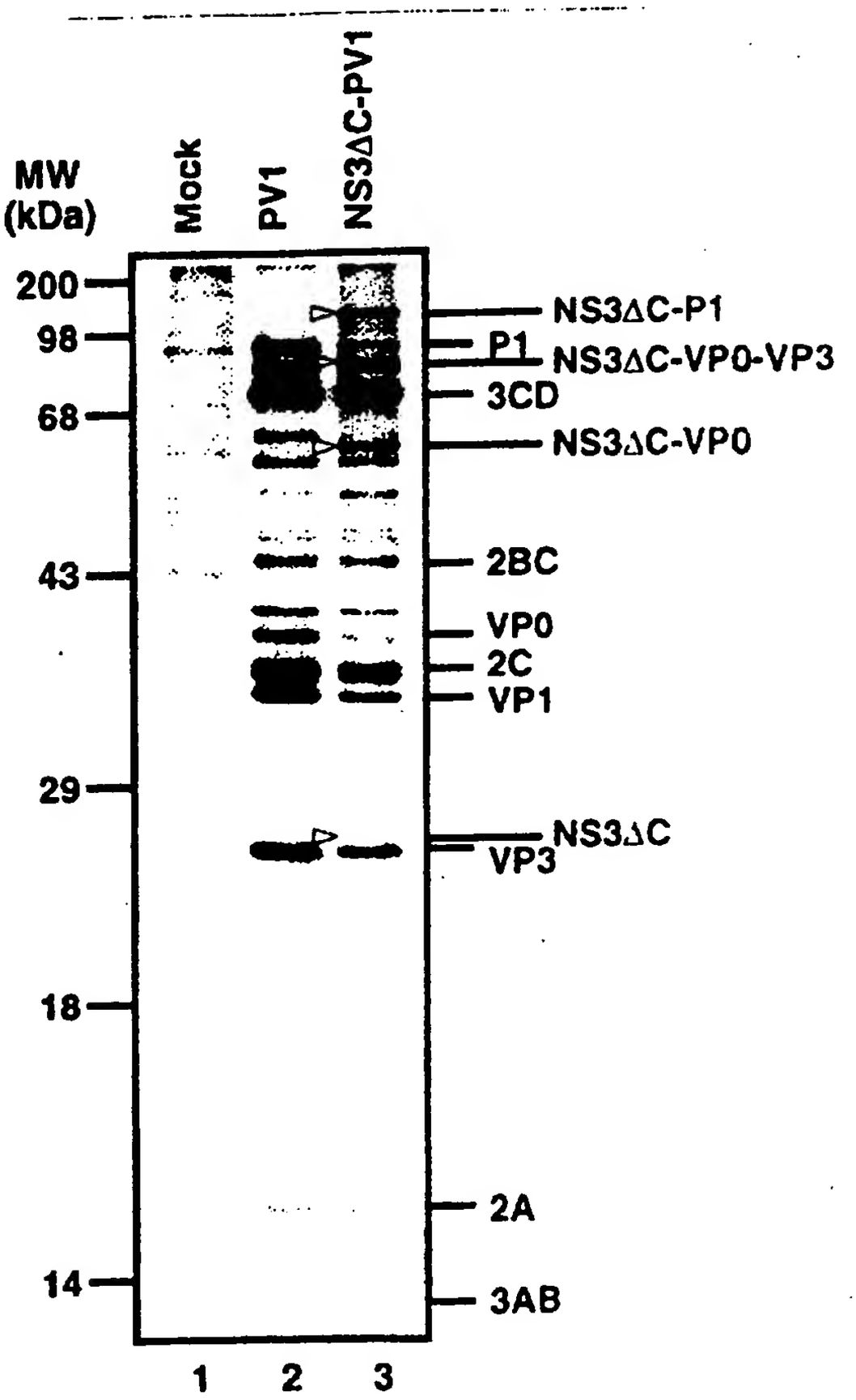

MW (kDa)
Mock
PV1
NS3ΔC-PV1
200
98
68
43
29
18
14
NS3ΔC-P1
P1
NS3ΔC-VP0-VP3
3CD
NS3ΔC-VP0
2BC
VP0
2C
VP1
NS3ΔC
VP3
2A
3AB
1
2
3

FIG. 4C

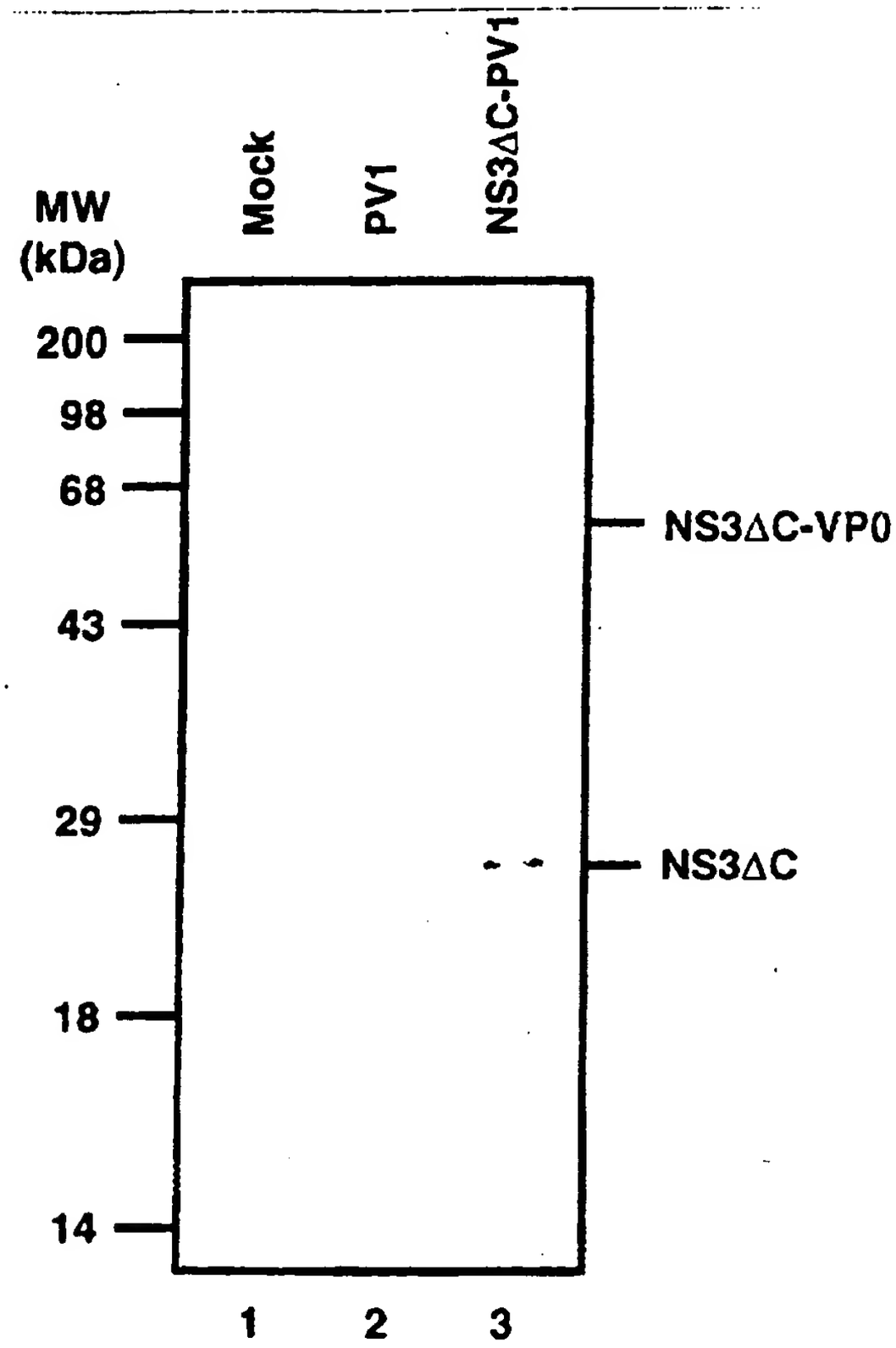


FIG. 5

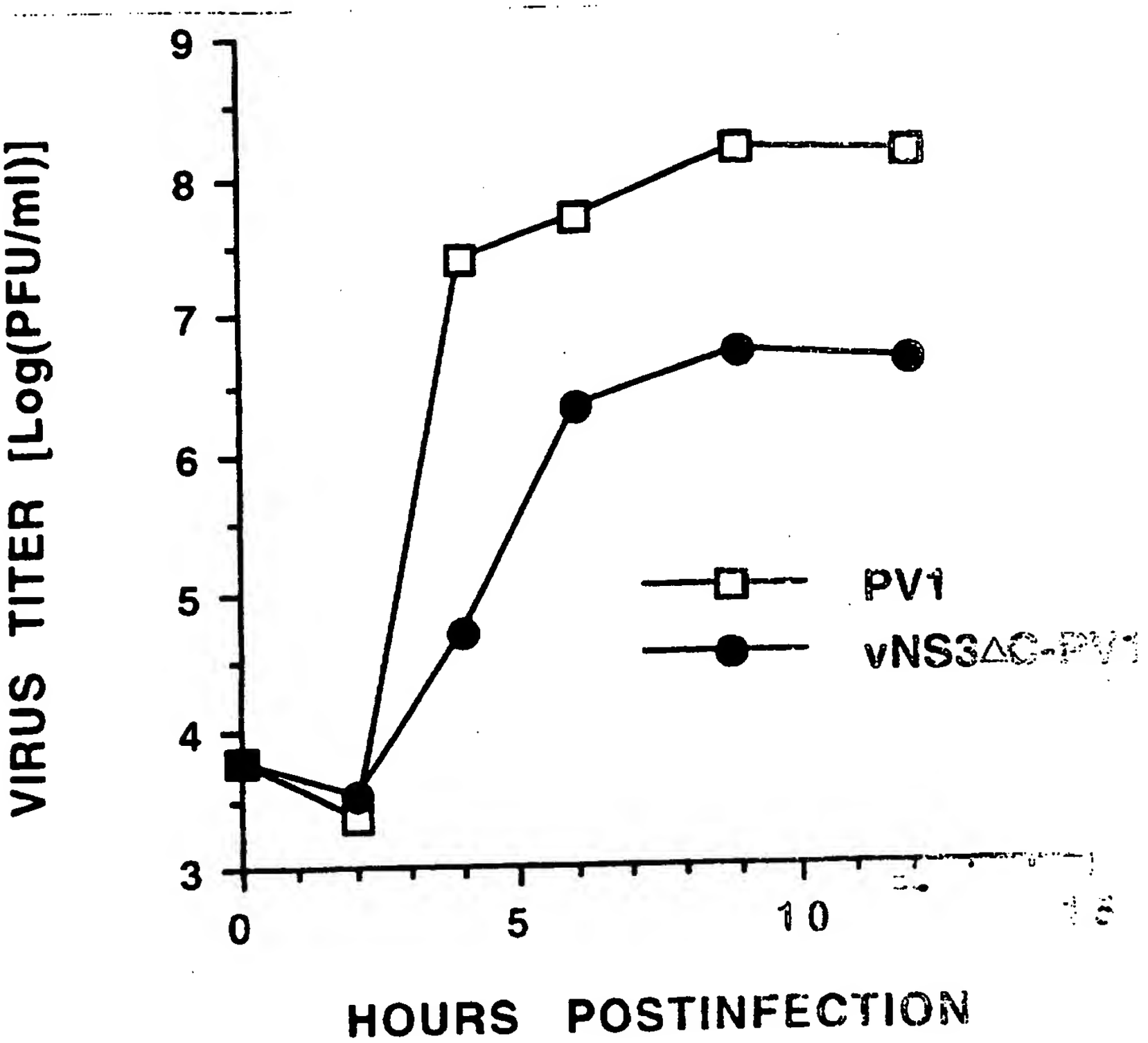

VIRUS TITER [Log(PFU/ml)]
9
8
7
6
5
4
3
0
5
10
HOURS POSTINFECTION
PV1
vNS3ΔC-PV1

FIG. 6A

TTAAAACAGCTCTGGGGTTGTACCCACCCCAGAGGCCCACGTGGCGGCTAGTACTCCGGT
ATTGCGGTACCCTTGTACGC
CTGTTTTATACTCCCTTCCCGTAACTTAGACGCACAAAACCAAGTTCAATAGAAGGGGG
TACAAACCAGTACCACCACGA
ACAAGCACTTCTGTTTCCCCGGTGATGTCGTATAGACTGCTTGCGTGGTTGAAAGCGACG
GATCCGTTATCCGCTTATGT
ACTTCGAGAAGCCCAGTACCACCTCGGAATCTTCGATGCGTTGCGCTCAGCACTCAACCC
CAGAGTGTAGCTTAGGCTGA
TGAGTCTGGACATCCCTCACCGGTGACGGTGGTCCAGGCTGCGTTGGCGGCCTACCTATG
GCTAACGCCATGGGACGCTA
GTTGTGAACAAGGTGTGAAGAGCCTATTGAGCTACATAAGAATCCTCCGGCCCCTGAAT
GCGGCTAATCCCAACCTCGGA
GCAGGTGGTCACAAACCAGTGATTGGCCTGTCGTAACGCGCAAGTCCGTGGCGGAACCGA
CTACTTTGGGTGTCCGTGTT
TCCTTTTATTTTATTGTGGCTGCTTATGGTGACAATCACAGATTGTTATCATAAAGCGA
AGACGGTATAGCTTGATATCG
AATTCCGGGGATCCTCTAGCATGGCCAGTCATCATCATCATCATCATGGAATTGTCGAC
AATTCCCCGGCCGATAGCCTT
GAAGGGCAGGGGTGGCAACTCCCCGCTCCCATCACGGCCTACTCCCAACAGACGCGGGGCC
TACTTGGTTGCATCATCAC
TAGCCTCACAGGCCGGGACAAGAACCAAGTCGAGGGGGAGGTTCAAGTGGTTTCCACCGC
AACACAATCTTTCCTGGCGA
CCTGCGTCAATGGCGCTTGGACTGTCTTCCATGGTGCCGGCTCAAAGACCCTAGCCGGCCC
AAAGGGGCCAATTACCCAA
ATGTACACCAATGTAGACCTGGACCTCGTCGGCTGGCAGGCACCCCCCGGGTCGCGTCCCC
TGACACCATGCACCTGCGG
CAGCTCAGACCTTTACTTGGTCACGAGACATGCTGATGTCATTCCGGTGCGCCGGCGGGG
CGACAGTAGGGGGAGCCTAC
CCTCTCCCAGACCAGTCTCCTACTTGAAGGGCTCCTCGGGTGGTCCACTGCTCTGCCCTTC
GGGGCACGCTGTTGGCATC
TTTCGGGCTGCTGTATGCACCCGGGGGGTTGCGAAGGCGGTGGACTTCATACCCGTTGAA
TCTATGGAAACTACTATGCG
GTCTCCGGTCTTCACAGATAACTCAACCGGAGGAGGAGGCGCGCAAGAGGAAGCCAGTGA
GGACGTCGTCTGCTGCGGAG
CTCAGGTTTCATCACAGAAAGTGGGCGCACATGAAAACTCAAATAGAGCGTATGGTGGT
TCTACCATTAATTACACCACC
ATTAATTATTATAGAGATTCAGCTAGTAACGCGGCTTCGAAACAGGACTTCTCTCAAGA
CCCTTCCAAGTTCACCGAGCC
CATCAAGGATGTCCTGATAAAAACAGCCCCAATGCTAAACTCGCCAAACATAGAGGCTT
GCGGGTATAGCGATAGAGTAC
TGCAATTAACACTGGGAAACTCCACTATAACCACACAGGAGGCGGCTAATTCAGTAGTC
GCTTATGGGCGTTGGCCTGAA
TATCTGAGGGACAGCGAAGCCAATCCAGTGGACCAGCCGACAGAACCAGACGTCGCTGCA
TGCAGGTTTTATACGCTAGA
CACCGTGTCTTGGACGAAAGAGTCGCGAGGGTGGTGGTGGAAGTTGCCTGATGCACTGAG
GGACATGGGACTCTTTGGGC
AAAATATGTACTACCACTACCTAGGTAGGTCCGGGTACACCGTGCATGTACAGTGTAAC
GCCTCCAAATTCCACCAGGGG
GCACTAGGGGTATTCGCCGTACCAGAGATGTGTCTGGCCGGGGATAGCAACACCACTACC
ATGCACACCAGCTATCAAAA
TGCCAATCCTGGCGAGAAAGGAGGCACTTTCACGGGTACGTTCACTCCTGACAACAACCA
GACATCACCTGCCCGCAGGT
TCTGCCCGGTGGATTACCTCCTTGGAAATGGCACGTTGTTGGGGAATGCCTTTGTGTTCC
CGCACCAGATAATAAACCTA
CGGACCAACAACTGTGCTACACTGGTACTCCCTTACGTGAACTCCCTCTCGATAGATAGT
ATGGTAAAGCACAATAATTG
GGGAATTGCAATATTACCATTGGCCCCATTAAATTTTGCTAGTGAGTCCTCCCCAGAGA
TTCCAATCACCTTGACCATAG

FIG. 6B

CCCCTATGTGCTGTGAGTTCAATGGATTAAGAAACATCACCCTGCCACGCTTACAGGGCC
TGCCGGTCATGAACACCCCT
GGTAGCAATCAATATCTTACTGCAGACAACTTCCAGTCACCGTGTGCGCTGCCTGAATT
TGATGTGACCCCACCTATTGA
CATACCCGGTGAAGTAAAGAACATGATGGAATTGGCAGAAATCGACACCATGATTCCCT
TTGACTTAAGTGCCACAAAAA
AGAACACCATGGAAATGTATAGGGTTCGGTTAAGTGACAAACCACATACAGACGATCCC
ATACTCTGCCTGTCACTCTCT
CCAGCTTCAGATCCTAGGTTGTCACATACTATGCTTGGAGAAATCCTAAATTACTACAC
ACACTGGGCAGGATCCCTGAA
GTTCACGTTTCTGTTCTGTGGATCCATGATGGCAACTGGCAAACTGTTGGTGTCATACG
CGCCTCCTGGAGCCGACCCAC
CAAAGAAGCGTAAGGAGGCGATGTTGGGAACACATGTGATCTGGGACATAGGACTGCAG
TCCTCATGTACTATGGTAGTG
CCATGGATTAGCAACACCACGTATCGGCAAACCATAGATGATAGTTTCACCGAAGGCGG
ATACATCAGCGTCTTCTACCA
AACTAGAATAGTCGTCCCTCTTTCGACACCCAGAGAGATGGACATCCTTGGTTTTGTGT
CAGCGTGTAATGACTTCAGCG
TGCGCTTGTTGCGAGATACCACACATATAGAGCAAAAAGCGCTAGCACAGGGGTTAGGT
CAGATGCTTGAAAGCATGATT
GACAACACAGTCCGTGAAACGGTGGGGGCGGCAACATCTAGAGACGCTCTCCCAAACACT
GAAGCCAGTGGACCAACACA
CTCCAAGGAAATTCCGGCACTCACCGCAGTGGAAACTGGGGCCACAAATCCACTAGTCCC
TTCTGATACAGTGCAAACCA
GACATGTTGTACAACATAGGTCAAGGTCAGAGTCTAGCATAGAGTCTTTCTTCGCGCGG
GGTGCATGCGTGACCATTATG
ACCGTGGATAACCCAGCTTCCACCACGAATAAGGATAAGCTTTTTGCAGTGTGGAAGAT
CACTTATAAAGATACTGTCCA
GTTACGGAGGAAATTGGAGTTCTTCACCTATTCTAGATTTGATATGGAACTTACCTTTG
TGGTTACTGCAAATTTCACTG
AGACTAACAATGGCCATGCATTAAATCAAGTGTACCAAATTATGTACGTACCACCAGGC
GCTCCAGTGCCCGAAAAATGG
GACGACTACACATGGCAAACCTCATCAAATCCATCAATCTTTTACACCTACGGGACAGCT
CCAGCCCGGATCTCGGTACC
GTATGTTGGTATTTCGAACGCCTATTCACACTTTTACGACGGTTTTTCCAAAGTACCAC
TGAAGGACCAGTCGGCAGCAC
TAGGTGACTCCCTTTATGGTGCAGCATCTCTAAATGACTTCGGTATTTTGGCTGTTAGA
GTAGTCAATGATCACAACCCG
ACCAAGGTCACCTCCAAAATCAGAGTGTATCTAAAACCCAAACACATCAGAGTCTGGTG
CCCGCGTCCACCGAGGGCAGT
GGCGTACTACGGCCCTGGAGTGGATTACAAGGATGGTACGCTTACACCCCTCTCCACCAA
GGATCTGACCACATATGGAT
TCGGACACCAAAACAAAGCGGTGTACACTGCAGGTTACAAAATTTGCAACTACCACTTG
GCCACTCAGGATGATTTGCAA
AACGCAGTGAACGTCATGTGGAGTAGAGACCTCTTAGTCACAGAATCAAGAGCCCAGGG
CACCGATTCAATCGCAAGGTG
CAATTGCAACGCAGGGGTGTACTACTGCGAGTCTAGAAGGAAATACTACCCAGTATCCT
TCGTTGGCCCAACGTTCCAGT
ACATGGAGGCTAATAACTATTACCCAGCTAGGTACCAGTCCCATATGCTCATTGGCCAT
GGATTCGCATCTCCAGGGGAT
TGTGGTGGCATACTCAGATGTCACCACGGGGTGATAGGGATCATTACTGCTGGTGGAGA
AGGGTTGGTTGCATTTTCAGA
CATTAGAGACTTGTATGCCTACGAAGAAGAAGCCATGGAACAAGGCCTCACCAATTACA
TAGAGTCACTTGGGGCCGCAT
TTGGAAGTGGATTTACTCAGCAGATTAGCGACAAAATAACAGAGTTGACCAATATGGTG
ACCAGTACCATCACTGAAAAG
CTACTTAAGAACTTGATCAAGATCATATCCTCACTAGTTATTATAACTAGGAACTATGA
AGACACCACAACAGTGCTCGC

FIG. 6C

TACCCTGGCCCTTCTTGGGTGTGATGCTTCACCATGGCAGTGGCTTAGAAAGAAAGCATG
CGATGTTCTGGAGATACCTT
ATGTCATCAAGCAAGGTGACAGTTGGTTGAAGAAGTTTACTGAAGCATGCAACGCAGCT
AAGGGCCTGGAGTGGGTGTCA
AACAAAATCTCAAAATTCATTGATTGGCTCAAGGAGAAAATTATCCCACAAGCTAGAGA
TAAGTTGGAATTTGTAACAAA
ACTTAGACAACTAGAAATGCTGGAAAACCAAATCTCAACTATACACCAATCATGCCCTA
GTCAGGAACACCAGGAAATTC
TATTCAATAATGTCAGATGGTTATCCATCCAGTCTAAGAGGTTTGCCCCTCTTTACGCA
GTGGAAGCCAAAAGAATACAG
AAACTCGAGCATACTATTAACAACTACATACAGTTCAAGAGCAAACACCGTATTGAACC
AGTATGTTTGCTAGTACATGG
CAGCCCCGGAACAGGTAAATCTGTAGCAACCAACCTGATTGCTAGAGCCATAGCTGAAA
GAGAAAACACGTCCACGTACT
CGCTACCCCCGGATCCATCACACTTCGACGGATACAAACAACAGGGAGTGGTGATTATGG
ACGACCTGAATCAAAACCCA
GATGGTGCGGACATGAAGCTGTTCTGTCAGATGGTATCAACAGTGGAGTTTATACCACC
CATGGCATCCCTGGAGGAGAA
AGGAATCCTGTTTACTTCAAATTACGTTCTAGCATCCACAAACTCAAGCAGAATTTCCC
CCCCCACTGTGGCACACAGTG
ACGCGTTAGCCAGGCGCTTTGCGTTCGACATGGACATTCAGGTCATGAATGAGTATTCT
AGAGATGGGAAATTGAACATG
GCCATGGCTACTGAAATGTGTAAGAACTGTCACCAACCAGCAAACTTTAAGAGATGCTG
TCCTTTAGTGTGTGGTAAGGC
AATTCAATTAATGGACAAATCTTCCAGAGTTAGATACAGTATTGACCAGATCACTACAA
TGATTATCAATGAGAGAAACA
GAAGATCCAACATTGGCAATTGTATGGAGGCTTTGTTTCAAGGACCACTCCAGTATAAA
GACTTGAAAATTGACATCAAG
ACGAGTCCCCCTCCTGAATGTATCAATGACTTGCTCCAAGCAGTTGACTCCCAGGAGGTG
AGAGATTACTGTGAGAAGAA
GGGTTGGATAGTTAACATCACCAGCCAGGTTCAAACAGAAAGGAACATCAACAGGGCAA
TGACAATTCTACAAGCGGTGA
CAACCTTCGCCGCAGTGGCTGGAGTTGTCTATGTCATGTATAAACTGTTTGCTGGACACC
AGGGAGCATACACTGGTTTA
CCAAACAAAAAACCCAACGTGCCCACCATTCGGACAGCAAAGGTACAAGGACCAGGGTTC
GATTACGCAGTGGCTATGGC
TAAAAGAAACATTGTTACAGCAACTACTAGCAAGGGAGAGTTCACTATGTTAGGAGTCC
ACGACAACGTGGCTATTTTAC
CAACCCACGCTTCACCTGGTGAAAGCATTGTGATCGATGGCAAAGAAGTGGAGATCTTG
GATGCCAAAGCGCTCGAAGAT
CAAGCAGGAACCAATCTTGAAATCACTATAATCACTCTAAAGAGAAATGAAAAGTTCAG
AGACATTAGACCACATATACC
TACTCAAATCACTGAGACAAATGATGGAGTCTTGATCGTGAACACTAGCAAGTACCCCA
ATATGTATGTTCCTGTCGGTG
CTGTGACTGAACAGGGATATCTAAATCTCGGTGGGCGCCAAACTGCTCGTACTCTAATG
TACAACTTTCCAACCAGAGCA
GGACAGTGTGGTGGAGTCATCACATGTACTGGGAAAGTCATCGGGATGCATGTTGGTGG
GAACGGTTCACACGGGTTTGC
AGCGGCCCTGAAGCGATCATACTTCACTCAGAGTCAAGGTGAAATCCAGTGGATGAGAC
CTTCGAAGGAAGTGGGATATC
CAATCATAAATGCCCCGTCCAAAACCAAGCTTGAACCCAGTGCTTTCCACTATGTGTTTG
AAGGGGTGAAGGAACCAGCA
GTCCTCACTAAAAACGATCCCAGGCTTAAGACAGACTTTGAGGAGGCAATTTTCTCCAA
GTACGTGGGTAACAAAATTAC
TGAAGTGGATGAGTACATGAAAGAGGCAGTAGACCACTATGCTGGCCAGCTCATGTCAC
TAGACATCAACACAGAACAAA
TGTGCTTGGAGGATGCCATGTATGGCACTGATGGTCTAGAAGCACTTGATTTGTCCACC
AGTGCTGGCTACCCTTATGTA

FIG. 6D

GCAATGGGAAAGAAGAAGAGAGACATCTTGAACAAACAAACCAGAGACACTAAGGAAA
TGCAAAAACTGCTCGACACATA
TGGAATCAACCTCCCACTGGTGACTTATGTAAAGGATGAACTTAGATCCAAAACAAAGG
TTGAGCAGGGGAAATCCAGAT
TAATTGAAGCTTCTAGTTTGAATGACTCAGTGGCAATGAGAATGGCTTTTGGGAACCTA
TATGCTGCTTTTCACAAAAAC
CCAGGAGTGATAACAGGTTCAGCAGTGGGGTGCGATCCAGATTTGTTTTGGAGCAAAAT
TCCGGTATTGATGGAAGAGAA
GCTGTTTGCTTTTGACTACACAGGGTATGATGCATCTCTCAGCCCTGCTTGGTTCGAGGC
ACTAAAGATGGTGCTTGAGA
AAATCGGATTCGGAGACAGAGTTGACTACATCGACTACCTAAACCACTCACACCACCTGT
ACAAGAATAAAACATACTGT
GTCAAGGGCGGTATGCCATCTGGCTGCTCAGGCACTTCAATTTTTAACTCAATGATTAA
CAACTTGATTATCAGGACACT
CTTACTGAAAACCTACAAGGGCATAGATTTAGACCACCTAAAAATGATTGCCTATGGTG
ATGATGTAATTGCTTCCTACC
CCCATGAAGTTGACGCTAGTCTCCTAGCCCAATCAGGAAAAGACTATGGACTAACTATG
ACTCCAGCTGACAAATCAGCT
ACATTTGAAACAGTCACATGGGAGAATGTAACATTCTTGAAGAGATTCTTCAGGGCAGA
CGAGAAATACCCATTTCTTAT
TCATCCAGTAATGCCAATGAAGGAAATTCATGAATCAATTAGATGGACTAAAGATCCTA
GGAACACTCAGGATCACGTTC
GCTCTCTGTGCCTTTTAGCTTGGCACAATGGCGAAGAAGAATATAACAAATTCCTAGCT
AAAATCAGGAGTGTGCCAATT
GGAAGAGCTTTATTGCTCCCAGAGTACTCAACATTGTACCGCCGTTGGCTTGACTCATT
TTAGTAACCCTACCTCAGTCG
AATTGGATTGGGTCATACTGTTGTAGGGGTAAATTTTTCTTTAATTCGGAGGAAAAAA
AAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACGGa
attAATTCTTGAAGACGAAAGGG
CCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGT
CAGGTGGCACTTTTCGGGGAA
ATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTC
ATGAGACAATAACCCTGATAA
ATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCT
TATTCCCTTTTTTGCGGCATT
TTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATC
AGTTGGGTGCACGAGTGGGTT
ACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGT
TTTCCAATGATGAGCACTTTT
AAAGTTCTGCTATGTGGCGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGT
CGCCGCATACACTATTCTCA
GAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAG
TAAGAGAATTATGCAGTGCTG
CCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGA
AGGAGCTAACCGCTTTTTTG
CACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCC
ATACCAAACGACGAGCGTGA
CACCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACT
TACTCTAGCTTCCCGGCAAC
AATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTT
CCGGCTGGCTGGTTTATTGCT
GATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGAT
GGTAAGCCCTCCCGTATCGT
AGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTG
AGATAGGTGCCTCACTGATTA
AGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACT
TCATTTTTAATTTAAAAGGATC

FIG. 6E

TAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTT
CCACTGAGCGTCAGACCCCGT
AGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGC
AAACAAAAAAACCACCGCTAC
CAGCTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTC
AGCAGAGCGCAGATACCAAA
TACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCC
TACATACCTCGCTCTGCTAA
TCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAA
GACGATAGTTACCGGATAAG
GCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACC
TACACCGAACTGAGATACCT
ACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCC
GGTAAGCGGCAGGGTCGGAA
CAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCG
GGTTTCGCCACCTCTGACTT
GAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAAC
GCGGCCTTTTTACGGTTCCT
GGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGA
TAACCGTATTACCGCCTTTG
AGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGG
AAGCGGAAGAGCGCCTGATG
CGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGT
ACAATCTGCTCTGATGCCGC
ATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCATGGCTGCGCCCCGA
CACCCGCCAACACCCGCTGA
CGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCC
GGGAGCTGCATGTGTCAGA
GGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGT
CGTGAAGCGATTCACAGATG
TCTGCCTGTTCATCCGCGTCCAGCTCGTTGAGTTTCTCCAGAAGCGTTAATGTCTGGCTT
CTGATAAAGCGGGCCATGTT
AAGGGCGGTTTTTTCCTGTTTGGTCACTTGATGCCTCCGTGTAAGGGGGAATTTCTGTT
CATGGGGGTAATGATACCGAT
GAAACGAGAGAGGATGCTCACGATACGGGTTACTGATGATGAACATGCCCGGTTACTGG
AACGTTGTGAGGGTAAACAAC
TGGCGGTATGGATGCGGCGGGACCAGAGAAAAATCACTCAGGGTCAATGCCAGCGCTTCG
TTAATACAGATGTAGGTGTT
CCACAGGGTAGCCAGCAGCATCCTGCGATGCAGATCCGGAACATAATGGTGCAGGGCGCT
GACTTCCGCGTTTCCAGACT
TTACGAAACACGGAAACCGAAGACCATTCATGTTGTTGCTCAGGTCGCAGACGTTTTGC
AGCAGCAGTCGCTTCACGTTC
GCTCGCGTATCGGTGATTCATTCTGCTAACCAGTAAGGCAACCCCGCCAGCCTAGCCGGG
TCCGCTCTCCCTTATGCGAC
TCCTGCATTAGGAAGCAGCCCAGTAGTAGGTTGAGGCCGTTGAGCACCGCCGCCGCAAGG
AATGGTGCATGCAAGGAGAT
GGCGCCCAACAGTCCCCCGGCCACGGGGCCTGCCACCATACCCACGCCGAAACAAGCGCTC
ATGAGCCCGAAGTGGCGAG
CCCGATCTTCCCCATCGGTGATGTCGGCGATATAGGCGCCAGCAACCGCACCTGTGGCGCC
GGTGATGCCGGCCACGATG
CGTCCGGCGTAGAGGATCCCGCGAAATTAATACGACTCACTATAGG

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 97/00120

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 N 15/51; C 12 Q 1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 12 N 15/51; C 12 Q 1/70

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Patent Abstracts of Japan, Vol.16, No.419 (C-981), 1992, Kokai No. 4-144 686 (KUNITADA SHIMOTOONO), abstract.	1
A	WO 92/08 734 A1 (CHIRON CORPORATION) 29 May 1992 (29.05.92), claims 1-6.	1

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 September 1997 (01.09.97)	05 September 1997 (05.09.97)
Name and mailing address of the ISA/ AT AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer Wolf Telephone No. 1/53424/436

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 97/00120

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 9208734	29-05-92	AU A1 90267/91	11-06-92
		AU B2 668078	26-04-96
		CA AA 2095521	09-05-92
		CZ A3 9300824	13-04-94
		EP A1 556292	25-08-93
		EP A4 556292	18-01-95
		FI A0 932025	05-05-93
		FI A 932025	07-06-93
		FI A 971702	21-04-97
		FI A0 971702	21-04-97
		HU A0 9301336	28-10-93
		HU A2 66063	28-09-94
		JP T2 6504431	26-05-94
		NO A0 931680	07-05-93
		NO A 931680	28-06-93
		NO A 972213	14-05-97
		NO A0 972213	14-05-97
		PT A 99466	30-10-92